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The genomic organization of the open reading frame of the red pigment concentrating hormone gene in the blue crab *Callinectes sapidus*

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Abstract

The open reading frame (ORF) of the gene for the precursor of the octapeptide Red Pigment Concentrating Hormone (RPCH) from the blue crab *Callinectes sapidus* was cloned by PCR with oligonucleotides targeted to the initiation and the end of the translation coding sequences. A 272 bp intron was characterized between nucleotides 343 and 344 of the reported cDNA, present in the region coding for the last amino acids of the precursor related peptide of RPCH. The intron genomic structure here described is similar to that reported for the gene coding for the Adipokinetic Hormone (AKH) of the grasshopper *Schistocerca nitans.*

Keywords: Crab; Crustacean; Gene; Intron; Invertebrate; Neurohormone; Neuropeptide; Peptide; Red Pigment Concentrating Hormone.

1. Introduction

The X organ-sinus gland system in the crustacean eyestalk secretes various neuropeptides. Their chemical structure suggests the existence of at least three families [1–4]; one of them is composed by four members: the Crustacean Hyperglycemic Hormone (CHH), the Molt-Inhibiting Hormone (MIH), the Gonad-Inhibiting Hormone (GIH), and the Vitellogenesis-Inhibiting Hormone (VIH); all of them with more than 70 residues. These peptides show considerable structural similarities among them, and some biological cross-reactivity. However, ample variations in structure have also been demonstrated for each hormone among different crustacean species [5].

The other two families correspond to the chromatophorotropic hormones represented by the Red Pigment Concentrating Hormone (RPCH) family and the Pigment Dispersing Hormone (PDH) family. PDH is an octadecapeptide capable of inducing dispersion of pigment granules in tegumentary chromatophores and retinal cells [6]. Its structure shows a certain degree of variability among crustacean groups [2,4]. In turn, RPCH is an octapeptide, with a functional role opposite to that of PDH i.e. it elicits aggregation of pigment granules in the same target cells [3,6,7].

The structure of RPCH is closely related to that of a neuropeptide family known as Adipokinetic Hormone (AKH) which is amply distributed among insects and partakes in the regulation of lipid and carbohydrate metabolism [8]. Given their structural similarities, the crustacean RPCH and the insect AKH are commonly considered as a single AKH/RPCH family. While only one structure is known for RPCH in all species so far studied [9], (PGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) there are three known variants to AKH structure among 33 structures identified in 75 insect species [8,10] for details see [10]: AKH I (PGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2), AKH II (PGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH_2), and AKH III (PGlu-Leu-Asn-Phe-Thr-Pro-Trp-NH_2). Another member of the family is the Hypertrehalosemic Hormone (HTH), which stimulates the synthesis of Trehalose, the main blood carbohydrate in many insect species. It is a decapeptide with the structure: Pro-Glu-Val-Asp-Phe-Ser-Pro-Gly-Trp-Gly-Thr-NH_2 [11]. RPCH is also related to the APGWamide neuropeptides in mollusks [12–14], which share the same amino acid residues with the minimal active unit of RCPH [7,9,13].

Little is known about the biosynthesis of the AKH/RPCH peptides. Two cDNAs have been characterized for

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the precursors of AKH I and II in *Schistocerca gregaria* [15,16] and *Schistocerca nitans* [17], and three cDNAs in *Locusta migratoria* [18]. Only one cDNA appears to be present in the pre-pro-AKH of *Manduca sexta* [19], *Drosophila melanogaster* [20] and, pre-pro-HTH in *Blaberus discoidalis* [21]. For RPCH, only the cDNAs of *Carcinus maenas* [22], and *Callinectes sapidus* [23], have been identified. While the genes coding for AKH in *M. sexta* [19], *D. melanogaster* [20] and *S. nitans* [24], have already been cloned, no information is available on the gene coding for the Pre-Pro-RPCH.

In this paper, we present the complete gene structure for the open reading frame coding for the RPCH precursor in the blue crab *Callinectes sapidus.* The elements of its intron are compared to those present in other genes of the precursors of the AKH/RPCH family, as well as with its possible relation with those of the APGWamide family.

2. Materials and methods

2.1. Isolation of DNA and RNA from C. sapidus

Genomic DNA from crab muscle was purified using the DNAzol reagent (Gibco-BRL) and RNase A treatment, as described by the manufacturer. To eliminate RNase A, DNA preparations were incubated with Proteinase K (100 μg/ml) at 42°C for 30 min, phenol-chloroform extracted, and ethanol precipitated.

Total RNA from crab muscle (for negative controls of RPCH expression) and nervous tissue from eyestalk was purified essentially as described by Chomczynski and Sacchi [25]. To avoid coprecipitation of retinal pigments, tissue lysates were subjected to two rounds of guanidine isothiocyanate-phenol-cloroform extractions followed by 2-propanol precipitation. Centrifugation steps were at 17600 g for 45 min. All procedures were carried out at 4°C. Poly A^+ RNA was purified with Mini Oligo (dT) Cellulose Spin Columns (5 Prime $\rightarrow$ 3 Prime) following the instructions of the manufacturer. DNA and RNA were quantitated by absorption at 260 nm.

2.2. PCR and RT-PCR for the RPCH precursor

500 ng of poly A^+RNA from eyestalk and muscle of *C. sapidus* were used for cDNA synthesis with the enzyme Superscript II (Gibco) at 55°C for 55 min, followed by RNAase H (Gibco) digestion according to the instructions of the manufacturer.

ORF amplification of the RPCH precursor was from both, genomic DNA and cDNA. Synthesis of primers was targeted to the ORF of the reported RPCH cDNA [23]. Reactions were set as follows: 10 nM of UJ (5′ATG GTT CGC AGA TCT GGA GTG 3′; nucleotides 65–86 in the cDNA; [23]), and LJ (5′ GTC TCG GCT CAG CTT AAC TTC TC 3′; nucleotides 394–374 in the cDNA; [23]) primers, 0.1 μg of genomic DNA or 1/25 of the reverse transcription reaction in the presence of 20 mM Tris-HCl at (pH 9.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 5 U of *Taq* DNA polymerase (GIBCO). Samples were incubated at 95°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min, for 30 cycles.

2.3. Characterization of PCR products

Amplified products were tested by nested PCR, they were set and incubated as described above, except that 20 ng of purified products from the amplification reactions replaced template DNA, and the primers were LJ and NJ (5′CTC AGC CCA GGT ACT CCT GAT C3′, positions 132–155 [23]) which span the last codons of the signal peptide and the first codons of the active peptide, respectively. Additional testing of the amplified products was carried out by restriction mapping with *Bam* HI, *Dde* I, *Hind* III and *Sau* 3AI (NEB) endonucleases. All PCR products were cloned in BlueScript SK. Sequencing was performed with thermo sequence (Amersham) and an Automatic Sequenator (Mod. ABI PRISM 310 Genetic Analyzer; Perkin Elmer) following the instructions of the manufacturer. Further analysis of the sequences was carried out with the package of GCG programs. All cloning experiments were repeated 3 times.

3. Results

PCR was used to amplify the genomic sequence coding for the RPCH precursor of *C. sapidus.* A product of 600 bp was amplified from genomic DNA using UJ and LJ primers, targeted to the ORF of the RPCH precursor (Fig. 1). The cDNA product obtained from RT-PCR with the same set of primers was of 331 bp. As expected, no product was amplified from crab muscle mRNA. These results suggested the presence of one or more introns within the genomic DNA of *C. sapidus.*

To test this possibility, nested PCR experiments were carried out using both, genomic DNA and mRNA templates, with the NJ and LJ set of primers. Genomic DNA rendered a 515 bp product, whereas the PCR product of mRNA was of 265 bp (Fig. 1). All the PCR products were subjected to restriction analysis to verify the presence of a single product; this would indicate the existence of only one RPCH gene (Fig. 2). The 126 bp *Bam* HI fragment present in the cDNA was absent in the genomic DNA, appearing instead a band of 398 bp. Likewise, the *Dde* I band of 85 bp in cDNA was absent in genomic DNA, and instead a band of 357 bp appeared (Fig. 2). Further restriction analysis showed intron specific *Hind* III and *Sau*3AI sites (see Fig. 3). These results confirmed the presence of a stretch of 272 bp of DNA inserted toward the 3′ end in the ORF genomic DNA of the RPCH (Fig. 2), and supported the notion of a

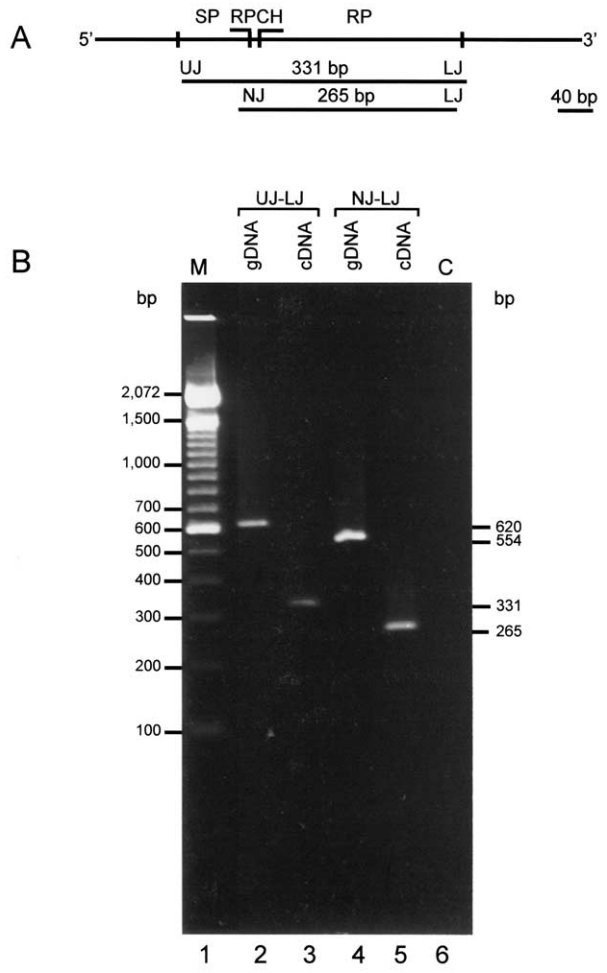

Fig. 1. PCR and RT-PCR of the ORF for the RPCH precursor. A. The top line shows a schematic representation of the cDNA structure of the RPCH precursor, and its domains coding for the signal peptide (SP), active peptide (RPCH) and RPCH-related peptide (RP). The lines underneath show DNA sizes of the expected products from RT-PCR and nested PCR experiments, using UJ-LJ and NJ-LJ as sets of primers, respectively. B. DNA products separated in a 1.5% agarose gel: lane 1, 100 bp DNA ladder (M); lanes 2 and 3, PCR and RT-PCR reactions using the set of primers UJ-LJ; lanes 4 and 5, nested PCR from genomic DNA and cDNA amplifications, using the set of primers NJ-LJ; lane 6 RT-PCR reaction of muscle mRNA using the set of primers UJ-LJ.

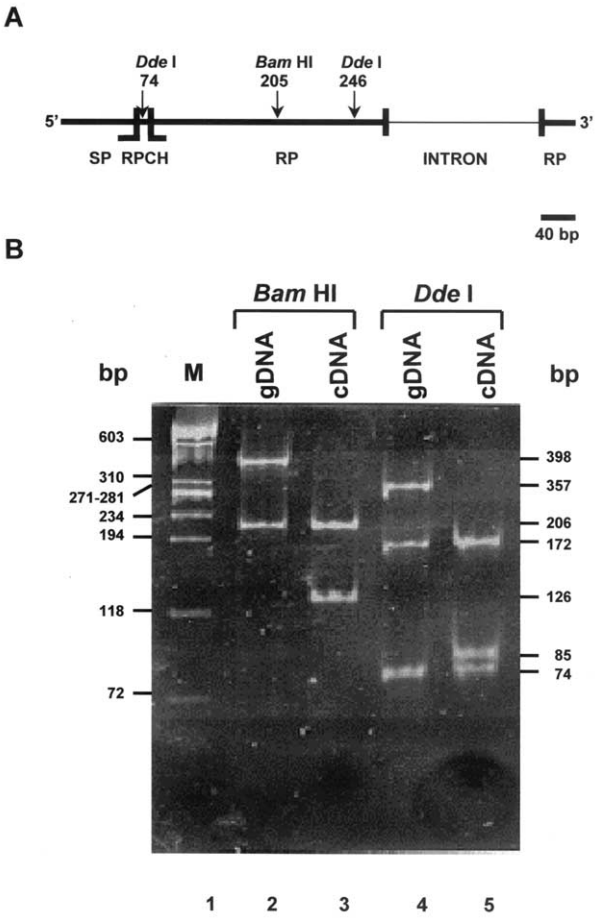

Fig. 2. Restriction analysis of PCR products amplified from the ORF of the RPCH precursor. A. Map of the RPCH precursor gene as deduced from Fig. 3. Numbers refer to restriction sites (see Fig. 3). SP, RPCH and RP are as in Fig. 1. B. Digestion products separated in a 7% polyacrylamide gel: lane 1, *Hae* III digest of phage ϕX174 DNA (M); lanes 2 and 3, *Bam* HI digests of PCR and RT-PCR products, respectively; lanes 4 and 5, *Dde* I digests of PCR and RT-PCR products, respectively. DNA size markers are shown in bp on the left margin. The size of the DNA restriction products is shown in bp on the right margin.

putative intervening element located in a position similar to those reported for the AKH genes of *S. nitans* [15].

To prove this sequence as a bona fide intron, the PCR product obtained from genomic DNA, was cloned and sequenced (Fig. 3). The sequence was compared to the whole GeneBank using BLAST analysis. *C. sapidus* genomic sequence of the RPCH precursor showed 98% homology with the cDNA of the RPCH precursor reported by Klein et al. [23], where the 5′ most 279 bp code for the signal peptide, the active RPCH, the dibasic cleavage site, and the first 54 amino acids of the RPCH-related peptide (RPCH-RP). The ORF in the genomic DNA is broken at position 279 (position 343 in the cDNA [23]) where the 272 bp intron begins. From position 280 to 286 an intronic consensus GTGAGT 5′ splice site is found. The branch site consensus (Y_{80} N Y_{80} Y_{87} P_{75} A Y_{95}) extends from position 519 to 525 followed by a pyrimidine-rich tract from position 534 to 547. The intron ends with the consensus NCAG 3′ splice site from positions 548 to 551 (Fig. 3). The ORF continues to the last 51 bp of the PCR product, coding for the last 16 amino acids of the RPCH-RP.

4. Discussion

The gene for the RPCH precursor of *C. sapidus* contains an intervening element in the RPCH-RP coding region. The intron we report here has so far not been identified in any other species. The *cis* elements of this intron are well conserved [26]. The presence of introns has been shown to occur in the genes coding for the AKH precursor of insects.

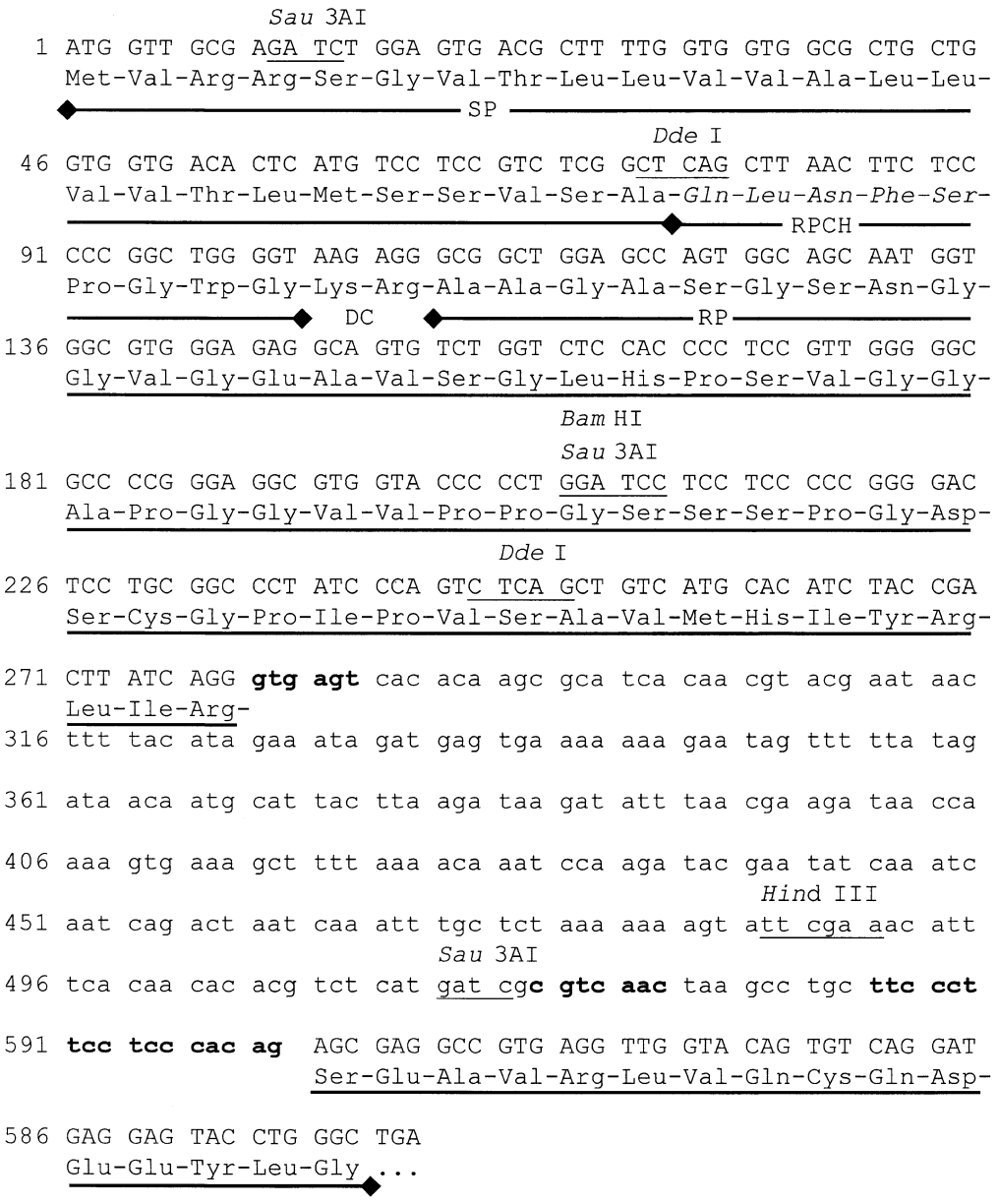

Fig. 3. Nucleotide sequence of the ORF of the RPCH precursor gene in *Callinectes sapidus.* The 272 bp intron (lower case letters) begins in nucleotide 279 and ends in nucleotide 551 of the cDNA. The donor and acceptor splice sites, the branch point sequence and poly pyrimidine tract consensus are shown in bold. Domains are labeled accordingly: SP, signal peptide; RPCH, active peptide; DC dibasic cleavage site; and RP, RPCH-related peptide. *Bam* HI, *Dde* I, *Hind* III, and *Sau* 3AI restriction sites are underlined. This sequence has been registered in GeneBank (accession number AF031654).

For instance, in *D. melanogaster* the intron interrupts the ORF of the active peptide [20]. In *S. nitans* the two genes coding for the AKH precursor (AKH I and AKH II), have two introns: one located in the 5′ UTR and the other interrupts the ORF of the AKH-RP. Thus, the location of the intron we report here, is similar to the second intron of the *S. nitans* AKH genes [24].

There are striking differences in the intron size among these genes, this feature is consistent with other introns of insects [27]. In *D. melanogaster* it is 67 bp long, and in *C. sapidus* it is 275 bp long; in *S. nitans* AKH I the intron is 2.1 kb long, and in *S. nitans* AKH II it spans for 15 kb (Fig. 4). In spite of this, all introns discussed here are phase 0 introns, i.e. they are located between two in-frame codons [28–30].

Notably, the intron structure reported here, is in good agreement with classic introns [31], and with those reported for the related genes in the AKH family. Donor and acceptor splice sites and branch point sequence are well conserved among these species (Fig. 4).

We identified, however, two discrete differences in intron structure. The poly pyrimidine tract of *C. sapidus* is not as well defined as in *D. melanogaster* and *S. nitans*. Concomitantly, the sequence between the branch point sequence

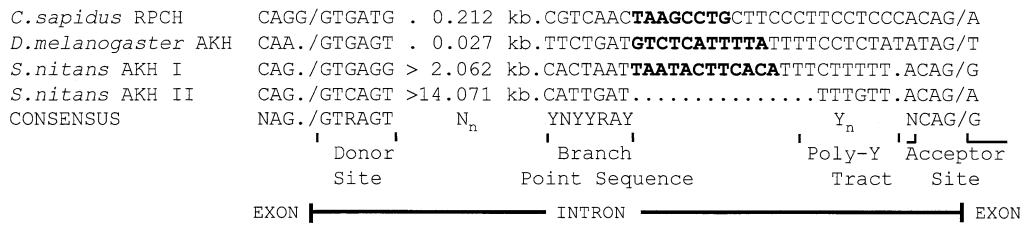

Fig. 4. Comparison of intron structures in the family of AKH/RPCH precursors. Donor and acceptor splice sites, branch point and poly pyrimidine tract consensus (R = purine; Y = pyrimidine) are well conserved [25], they differ mainly in intron length, poly pyrimidine tract definition, and the sequence between this tract and the branch point sequence (in bold).

and the poly pyrimidine tract is shorter in length and 50% GC-richer in *C. sapidus*, whereas in genes for *D. melanogaster* AKH and *S. nitans* AKH I they are slightly larger and richer in AT, resembling those of type III introns [32]. These sequences are absent in the intron of the *S. nitans* AKH II gene (Fig. 4). The biological relevance of these features is an open question.

This *C. sapidus* intron, albeit showing some differences with the AKH family in insects, bears important similarities in other parts of the RPCH gene. However, some important differences in the RPCH gene appear to exist among arthropods. An outstanding feature in the ORF in AKH genes is the way in which they are structured. While the ORF in AKH gene of *M. sexta* lacks introns [19], that of *D. melanogaster* has a 68 pb intron between the codons for the first and second codon of AKH. In turn, in *S. nitans*, the ORF in AKH I gene has an intron of 2.1 kb. The AKH II gene has the same structure as that of AKH I, but the intron is 15 kb long [24]. The size of the introns suggests the participation of different intronic and exonic elements in the splicing to form the precursor mRNA. Further studies of the gene structure of other members of AKH/RPCH gene family and their relations with those of the APGW-amide family, will shed light on the processes driving their evolution.

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